

Analysis of serum IgG against *Treponema pallidum* protein antigens in experimentally infected rabbits

JOHN F ALDERETE AND JOEL B BASEMAN

From the Department of Microbiology, University of Texas Health Science Center, San Antonio, Texas, USA

SUMMARY Sensitive and highly specific radioimmunoprecipitation and gel electrophoresis-fluorography were used to monitor the serum IgG response of experimentally infected rabbits against key protein antigens of *Treponema pallidum*. The sera from six rabbits challenged intradermally (ID) and six intratesticularly (IT) with *T pallidum* were examined over a period of four months. A specific antibody response to most treponemal surface proteins (including those ligands implicated as mediating host-cell-surface adherence) developed by about day 20 after inoculation. Maximum antibody concentrations against most of these immunogens were detected by day 50 in all animals irrespective of the route of inoculation. Differences as well as similarities in the IgG response to infection were observed among selected rabbits. Furthermore, the antibody reactivity of each animal was comparable to that of human sera from patients with secondary syphilis. Antibody directed against treponemal outer envelope proteins was detected by preferential agglutination of washed radiolabelled organisms with protein A-bearing *Staphylococcus aureus* in the presence of syphilitic rabbit sera.

Introduction

Although evidence supports an important role for humoral immunity in acquired resistance to syphilis,¹⁻¹¹ no protective treponemal immunogens have been identified. Information correlating host protection with the appearance of antibody directed against specific *Treponema pallidum* antigens would be helpful in understanding the progression of the disease and host susceptibility to infection.

Because of the complex interrelationship between virulent treponemes and host cells¹²⁻¹⁷ and the continuing difficulties in cultivating the pathogen *in vitro*, alternative methods are necessary for identifying critical treponemal components concerned in the pathogenesis of the disease. Recently, we demonstrated the usefulness of immunoprecipitation techniques for investigating treponemal surface ligands.¹³⁻¹⁸ We assessed the complicated interaction between treponemes and host macro-

molecules¹⁹ and monitored the reactivity of convalescent human syphilitic serum with radiolabelled *T pallidum* proteins.¹³ In this paper we analyse the kinetics of the IgG response to treponemal infection in the rabbit and characterise proteinaceous immunogens of *T pallidum* in relation to the development of experimental syphilis.

Materials and methods

BACTERIA

The Nichols strain of *T pallidum* was obtained from the Center for Disease Control, Atlanta, Georgia. Treponemes used in this study were freshly extracted from infected rabbit testes at peak orchitis and separated from host cellular contamination.^{19,20}

Motile freshly extracted treponemes were injected intradermally (ID) at duplicate sites on the shaved back of six rabbits in 0.1-ml volumes of graded doses containing 4×10^2 , 4×10^3 , and 4×10^5 treponemes.^{21,22} This procedure was adopted to obtain an antibody response against treponemal antigens under the conditions routinely used to estimate the efficacy of potential protective preparations by the size and severity of the resulting lesions.² Sites injected with 4×10^5 heat-killed organisms and with

Address for reprints: Dr J F Alderete, Department of Microbiology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284, USA

Accepted for publication 2 April 1981

filtered (0.22 μ) treponeme-free testicular extract from the same animal served as negative controls.

Six rabbits were also injected intratesticularly (IT) with approximately 5×10^7 organisms per testis and given 25 mg cortisone acetate daily from days 3 to 7 post-inoculation to facilitate the development of orchitis and reduce possible individual variations in the response. Peak orchitis was reached at day 10.

SERA

Whole blood, obtained from the ear vein of rabbits before and at various intervals after infection, was allowed to clot at room temperature for 60 minutes and then incubated for 60 minutes at 4°C before centrifugation. Human syphilitic sera (HSS) from the secondary stage of infection were obtained from S A Larsen (Center for Disease Control, Atlanta, Georgia, USA). All sera were stored at -70°C before use.

RADIOIMMUNOPRECIPITATION (RIP) ASSAY

Approximately 8×10^9 treponemes were freshly extracted from the testes of infected rabbits in 20 ml of a glucose-salts medium with reducing agents containing 10% fetal bovine serum (Grand Island Biological Co, Grand Island, NY, USA). These preparations were incubated with [³⁵S]-methionine (10 μ Ci/ml, New England Nuclear, sp act 950 Ci/mmol) for 20 hours in an air atmosphere at 37°C.^{18 19} After incubation treponemes were pelleted at 17 500 $\times g$ for 15 minutes, washed once in phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 4.6 mmol/l Na₂HPO₄, and 1.5 mmol/l KH₂PO₄) and stored at -70°C until required. Radiolabelled protein patterns of frozen *T pallidum* preparations were identical to those of freshly harvested organisms or treponemes radiolabelled for shorter times based on sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and radioimmunoprecipitation (RIP).

Frozen pellets containing 4×10^9 radiolabelled treponemes were suspended in 100 μ l of NET (150 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid, 50 mmol/l TRIS hydrochloride, pH 7.2) buffer containing 1 mmol/l phenylmethylsulfonyl-fluoride (PMSF; Sigma Chemical Co, St Louis, Missouri, USA). Ten microlitres of 1% SDS were added,¹³ and the suspension was gently homogenised until the treponemes were solubilised. Then 100 μ l of 10 mg/ml ovalbumin were added to combine with excess SDS. Finally, 100 μ l of 10% Triton X100 were introduced and the mixture incubated at 37°C for 10 minutes. The preparation was centrifuged over a 5% sucrose bed for 45 minutes at 150 000 $\times g$ using a Dupont-Sorvall AH650 rotor. The supernatant containing radiolabelled solubilised treponemes was

then diluted to a final volume of 1 ml in NET buffer.

Before specific RIP, the radiolabelled preparation was preadsorbed with formalin-fixed protein A-bearing *Staphylococcus aureus*²³ to remove non-specific binding proteins. Then 100 μ l of the adsorbed solubilised treponemal proteins were added to microfuge tubes containing 20 μ l of antiserum and incubated at 37°C for two hours. Finally, 50 μ l of 10% (v/v) fixed *Staph aureus*^{19 23} were added and incubation continued at room temperature for a further 30 minutes. The *Staph aureus*-adsorbed immune complexes were then sedimented in a Beckman Microfuge B miniature centrifuge and washed three times in NET 0.05% Triton X100 buffer. To the pellet were added 75 μ l of dissolving buffer (62.5 mmol/l TRIS hydrochloride, pH 6.8, 2% mercaptoethanol, 10% glycerol, 2% SDS, and 0.02% bromophenol blue as the tracking dye) and the samples boiled for three minutes. The *Staph aureus* were then removed by centrifugation and the supernatant containing radiolabelled treponemal proteins immediately loaded on SDS polyacrylamide slab gels.^{13 18 19} Electrophoresis was performed using 3% and 7.5% acrylamide respectively for stacking and separating gels. Gels were then fixed and processed for autoradiography.^{18 19 24}

ANTIBODY-MEDIATED AGGLUTINATION OF TREPONEMES

Protein A-bearing *Staph aureus* to be used as the immunoabsorbent were prepared.^{19 23} Washed radiolabelled treponemes pre-exposed to various test sera were agglutinated with *Staph aureus*, and the extent of agglutination of the treponeme-antibody-staphylococcal complex was expressed as counts per minute obtained with test sera subtracted from counts per minute using control sera.¹⁹ Control values never exceeded 30% of total counts per minute, and all sera were diluted 1/100 in NET buffer unless otherwise stated.

Results

ANTITREPONEMAL ANTIBODY RESPONSE

The early IgG response of rabbits challenged by different routes with *T pallidum* was examined to clarify the nature of the initial antibody response. Protein A-mediated immunoprecipitation of solubilised [³⁵S]-methionine-labelled treponemal proteins by early rabbit syphilitic sera (RSS) is shown in fig 1. At day 14 post-inoculation, antibodies against several treponemal antigens were present in the two representative rabbits infected ID (fig 1; A and B). By day 19 antibodies against proteins labelled 1, 2 (doublet), 3, and 6 for rabbit A and proteins 1, 2 (doublet), 3, 5, and 6 for rabbit B were present.

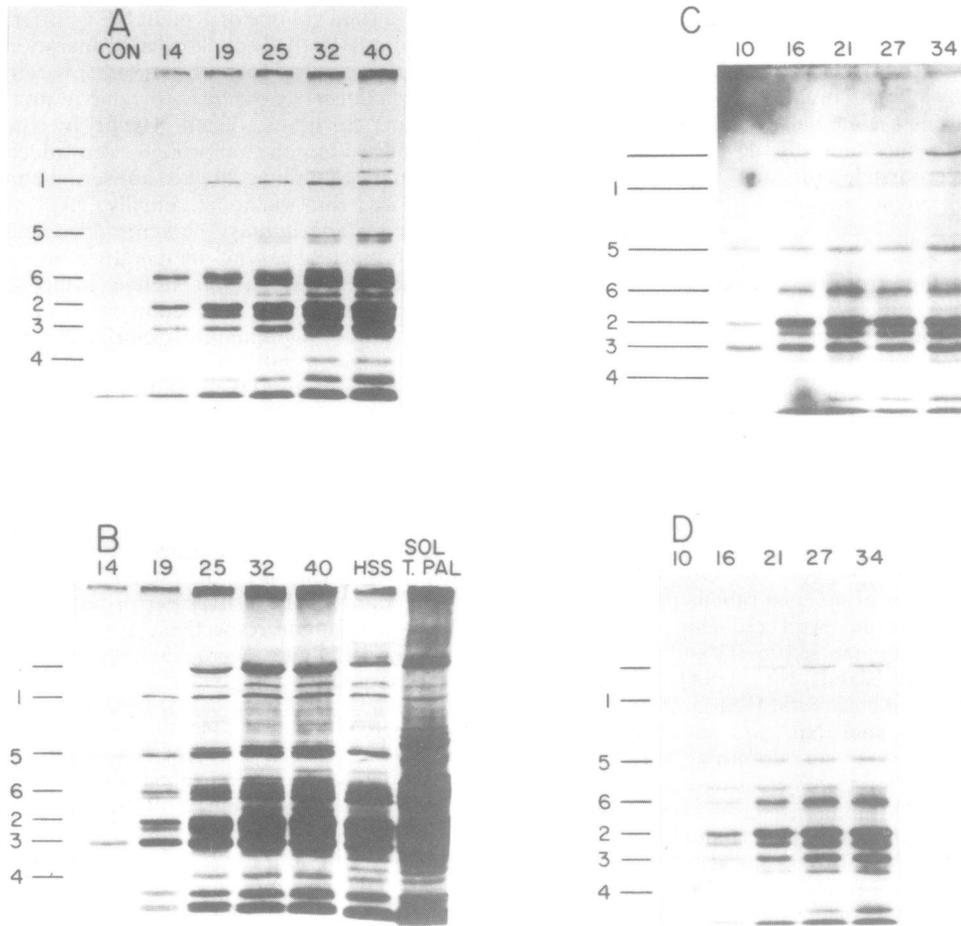


FIG 1 SDS-acrylamide fluorograms of [^{35}S]-methionine-labelled treponemal proteins (1, 2, 3, 4, 5, and 6) immunoprecipitated with rabbit and human syphilitic sera. Numbers refer to the day post-infection that each serum was collected from two representative rabbits challenged intradermally (A and B) and two intratesticularly (C and D). Human serum (HSS) from a patient with secondary syphilis is shown for comparison. The SOL T PAL profile represents the total solubilised treponemal proteins in the reaction mixture.

Rabbit B appeared to have mounted a stronger initial humoral response than rabbit A (fig 1). The concomitant increase in the intensity of these and other antigens as the infection progressed is interesting and indicative of increased IgG concentrations of the individual proteins.

The specificity of the antibody-antigen complexes is shown by the inability to immunoprecipitate radio-labelled treponemal proteins with control NRS obtained from these rabbits before infection. Proteins 1, 2, 3, 4, 5, and 6 with molecular weights of 89 500, 29 500, 25 500, 20 000, 59 000, and 42 500 daltons respectively have been shown to reside on the outer membrane of *T pallidum*, based on both

[^{125}I]-lactoperoxidase-catalysed iodination of intact organisms¹⁸ and trypsin sensitivity.¹⁹ Proteins 1, 2, and 3 have been implicated as the ligands responsible for the parasitism of host-cell surfaces.¹³

RIP profiles were determined in sera from rabbits infected IT under the conditions routinely used to cultivate treponemes. Examination of the early antibody response showed profiles similar to those obtained for ID-inoculated animals (fig 1; C and D). Sera reacted with proteins 2, 3, 5, and 6 by day 10 in rabbit C and by day 16 in rabbit D. Whereas both IT-challenged rabbits developed an orchitis on the same day the initial immune response by rabbit D lagged behind that of rabbit C. Only low concentrations of

IgG against protein 1, as well as against several other *T pallidum* proteins, were detected in both rabbits challenged IT. These data are from selected individual rabbits injected intradermally and intratesticularly. Sera from additional rabbits examined under similar conditions had protein profiles similar to those shown in fig 1.

CORRELATION OF LESION DEVELOPMENT AND RIP PROFILES

Because the degree and nature of lesion development are used as criteria in vaccine studies as well as for evidence of host immune mechanisms in syphilis, we attempted to correlate the development of lesions in the representative rabbits infected ID with the appropriate fluorograms. The lesions of rabbit A were less raised and somewhat flatter, and the margins not well defined, compared with those of rabbit B. The extent of the lesions in rabbit A never equalled that of rabbit B, in which caseous centres continued to erode producing the common crusted craters observed before regression of the lesions. These observations correspond directly with the fluorograms (fig 1; A and B). The more vigorous lesions in rabbit B were accompanied by a more pronounced antibody response compared with the protein profiles obtained from the sera of rabbit A (fig 1; A and B).

ANTIBODY RESPONSES AT LATER STAGES OF INFECTION

The extended nature of the antibody response to experimental syphilis in rabbits is shown in fig 2. Maximum reactivity of RSS with radiolabelled treponemal antigens occurred around day 40 for ID-inoculated rabbits (A and B) and day 48 for IT-inoculated rabbits (C and D) (fig 2). When the fluorograms of rabbits A and B are compared with those of rabbits C and D substantial differences in the kinetics of the antibody pattern against higher molecular-weight treponemal antigens are evident; a slower but more extensive humoral response to these antigens occurred after IT challenge (fig 2). A notable exception was the presence of antibody reactive with protein 1, which was detected in all animals. The intensity of the fluorogram for proteins 2 and 3 in all rabbits correlates with high antibody concentrations for these antigens and supports their possible immunological importance during this period of infection.

SPECIFIC ANTITREPONEMAL ANTIBODY CONCENTRATIONS

Because a strong humoral immune response occurred about day 40 and day 48 in ID-inoculated and IT-inoculated rabbits respectively and because of the

seemingly high titre of antibody against the putative ligands mediating attachment,¹³ we titrated antibody concentrations against proteins 1, 2, and 3 in the various test sera. A 1/100 final dilution of each syphilitic serum still resulted in significant reactivity against most treponemal antigens (fig 3). The fluorogram at the 1/1000 dilution indicates that several proteins, including 1, 2, and 3, were highly antigenic. IT challenge (rabbits B1 and B2) yielded higher concentrations of antibody than ID challenge (rabbits A1 and A2), based on spectrophotometric scanning and quantification of the fluorogram (fig 3). Additional experimentation, however, is required to correlate antigen load with antibody titre.

Finally, analysis of human syphilitic sera obtained during the secondary stage of infection (fig 3; C) resembled the profiles of both ID-infected and IT-infected rabbits. Heat-inactivation of each test serum did not alter the RIP profiles.

INTERACTION OF ANTITREPONEMAL ANTIBODIES WITH INTACT TREPONEMES

To determine the function of specific antibody in relation to the development of disease, we examined the reactivity of intact treponemes with the various test sera. Protein A-bearing *Staph aureus* was added to washed intact radiolabelled *T pallidum* which had been exposed to RSS antibody. Figure 4 shows agglutination of treponemes with the rabbit test sera and illustrates the considerable reactivity obtained for each serum irrespective of the route of inoculation. The increase in the extent of agglutination was directly related to raised antibody titres (figs 1 and 2), which is also consistent with the presence of high concentrations of specific antibody against the surface proteins of *T pallidum*.¹⁸

Discussion

In this study radioimmunoprecipitation and gel electrophoresis-fluorography were combined to examine the kinetics of the host antibody response to specific *T pallidum* antigens. A pronounced IgG response to numerous treponemal proteins, including proteins 1, 2, and 3, was detected. The latter reside on the outer envelope of *T pallidum*¹⁸ and are implicated as ligands mediating the attachment of the parasite to host cells.¹³ Their antigenic character and the time course of the early antibody response to these and other treponemal proteins were clearly demonstrated (figs 1 and 2).

The presence of IgG against treponemal surface proteins was examined by *Staph aureus*-mediated agglutination of intact treponemes pretreated with test sera (fig 4). Although these data indicate that antibodies against key components such as trepo-

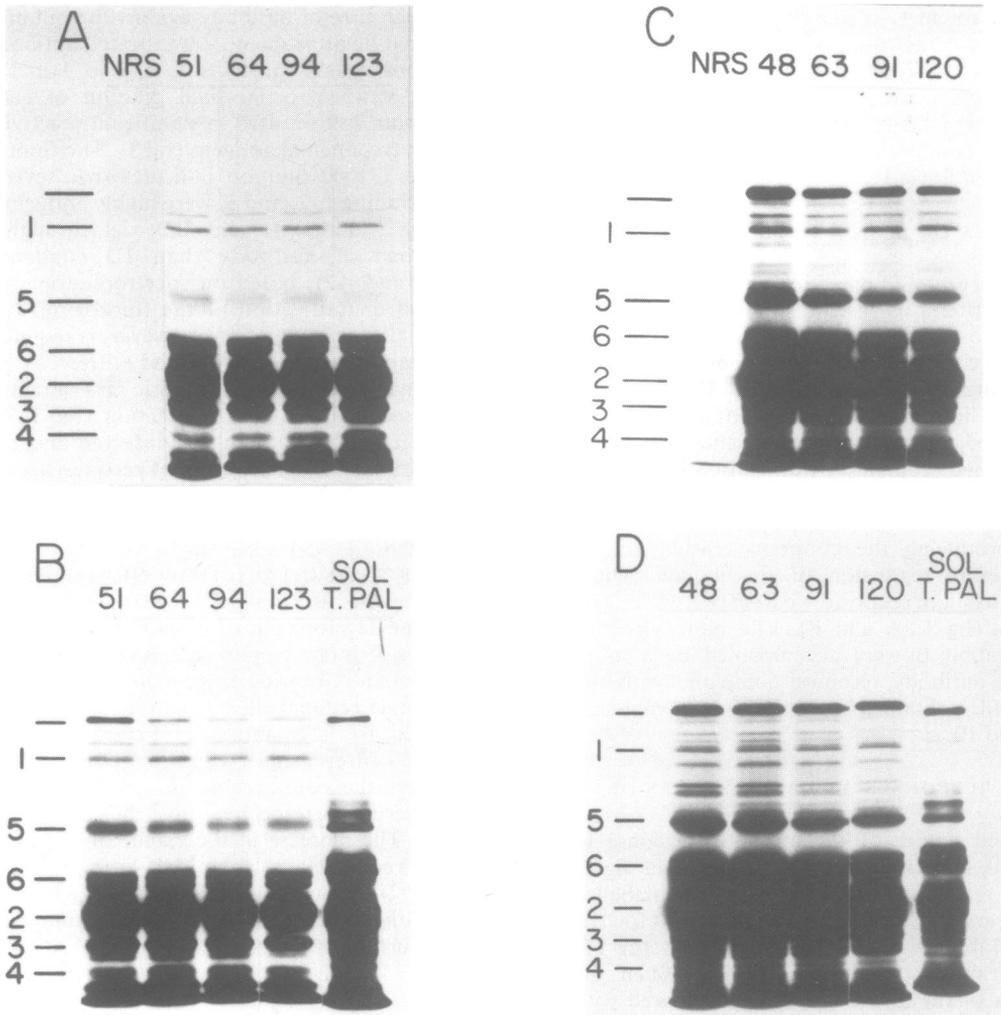


FIG 2 Radioimmunoprecipitation fluorograms of [^{35}S]-methionine-labelled treponemal proteins reactive with rabbit syphilitic sera. Numbers indicate the day post-infection when sera were collected from rabbits inoculated ID (A and B) and those inoculated IT (C and D). Control normal rabbit serum (NRS) was obtained from each animal before infection. The final serum dilution was 1/10 in NET buffer, and SOL T PAL represents the control profile of treponemal proteins used in the reaction mixture.

nemal ligands exist during infection, we have recently described the masking of the treponemal surface by host macromolecules, including non-specific IgG and IgM. Such serum proteins might compromise host defences against *T pallidum*^{19 25} by allowing certain organisms to persist in the presence of high concentrations of antitreponemal antibody. In support of this, IgG molecules were recently detected on the surface of virulent treponemes using antibody against both Fc and Fab components (Alderete and Baseman, unpublished data), suggesting a possible

role for blocking antibody in the aetiology of the infection.

Although the total contribution of either humoral or cellular immune mechanisms to overall immunity has not been determined, it is clear that cell-mediated immunity is altered during the early stages of infection.²⁶ The depression of normal lymphocyte responses, for example, to various activators by syphilitic serum^{27 28} and the inability of activated rabbit peritoneal macrophages to ingest motile treponemes anchored to the cell surface^{12 18 29} illustrate the

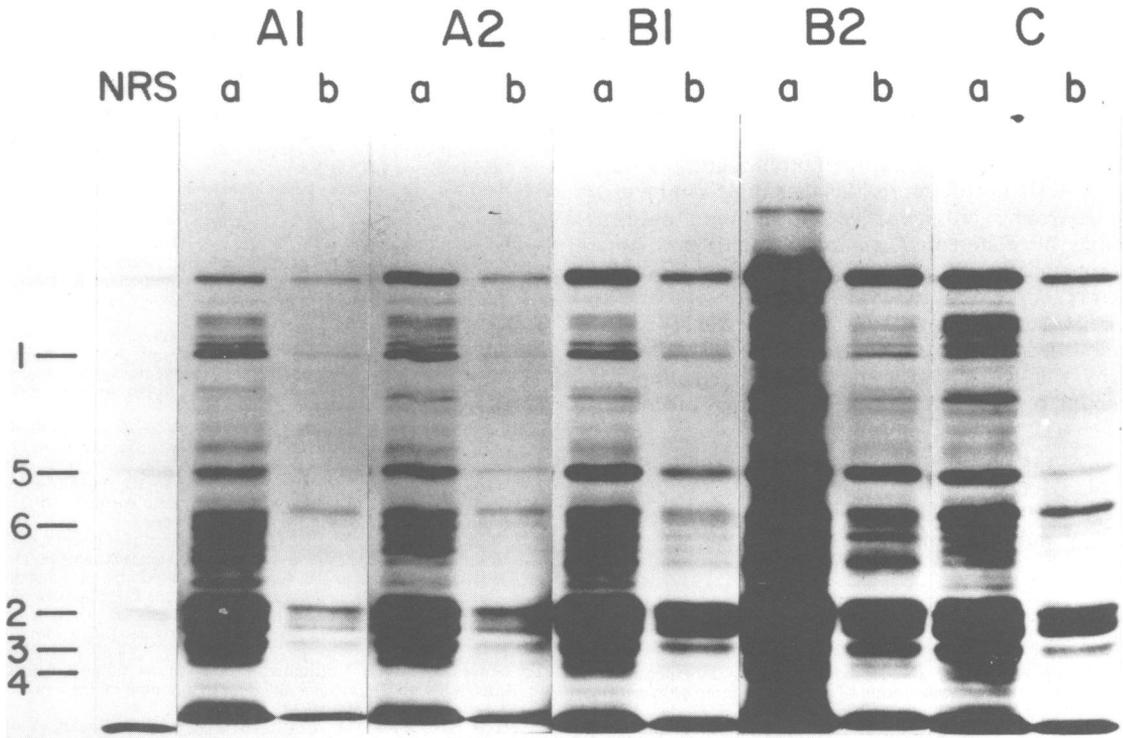


FIG 3 Titration of antitreponemal antibody in representative sera from two rabbits infected ID at day 40 (A1 and A2) and two challenged IT at day 48 (B1 and B2) and a patient with secondary syphilis (C) are shown. Final serum concentrations were 1/100 (a) and 1/1000 (b) in NET buffer. NRS or normal human serum diluted 1/10 served as control and similar results were obtained as illustrated by the NRS profile.

complex nature of cellular immune mechanisms in host defence against syphilis. Similarly, the correlation of antitreponemal antibody with protection has been hindered by the lack of information on the kinetics of antibody response to biologically important *T pallidum* antigens. Further analysis of model systems and the characterisation of chemical-antigenic properties of *T pallidum* are, therefore, essential for understanding disease pathogenesis.

The technology used here to characterise the humoral immune response in *T pallidum*-infected animals has several applications. The presence of antibody against major treponemal proteins can be determined and correlated with the progression of the disease. Sera from animals infected over prolonged periods contain high concentrations of IgG primarily against proteins 2 and 3 (Alderete and Baseman, unpublished observations). Such long-term analysis of RIP gel profiles from acute and con-

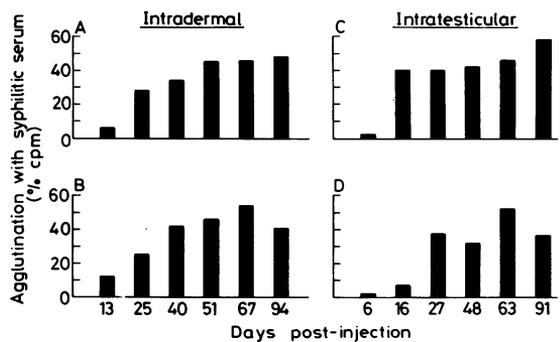


FIG 4 Agglutination of [³⁵S]-methionine-labelled and washed treponemes with protein A-bearing *Staph aureus* is shown. Treponemes were preincubated with each selected serum sample obtained at various days after infection from ID-inoculated rabbits (A and B) and IT-inoculated rabbits (C and D).

valescent individuals or animals immune to re-infection^{4 5} might provide insight into those host factors which determine susceptibility or resistance to treponemal infections. Relationships between specific antibody response and concomitant tissue damage may implicate antigen-antibody complexes which contribute to possible autoimmune reactions in the host. Furthermore, this technique can be used to determine cross-reactive or common antigens shared by different *T pallidum* strains and other treponemal species. A basis for identifying virulence factors or permitting an alternative approach to vaccine development or both could thus be established. We hope that the procedures used in this study will be useful in the analysis of a humoral immune response to the stresses of other infections.

This work was supported by Public Health Service grant AI-15036 and research career development award 1-K04-AI-00178 from the National Institute of Allergy and Infectious Diseases.

References

- Bishop NH, Miller JN. Humoral immunity in experimental syphilis. I The demonstration of resistance conferred by passive immunization. *J Immunol* 1976; **117**:191-6.
- Cannefax GR. Immunity in syphilis. *Br J Vener Dis* 1965; **41**:260-74.
- Miller JN. Immunity of experimental syphilis. V The immunogenicity of *Treponema pallidum* attenuated by γ -irradiation. *J Immunol* 1967; **99**:1012-6.
- Miller JN. Immunity in experimental syphilis. VI Successful vaccination of rabbits with *Treponema pallidum*, Nichols strains, attenuated by gamma-irradiation. *J Immunol* 1973; **110**:1206-15.
- Miller JN, Fazzan FP, Whang SJ. Studies on immunity in experimental syphilis. II *Treponema pallidum* immobilization (TPi) antibody and the immune response. *Br J Vener Dis* 1963; **39**:199-203.
- Perine PL, Weiser RS, Klebanoff SF. Immunity to syphilis: passive transfer in rabbits with hyperimmune serum. *Infect Immun* 1973; **8**:878-90.
- Podwinska J, Tyran B. Isolation and characterization of low-molecular weight antibodies for *Treponema pallidum*. *Arch Immunol Therap Experimentalis* 1979; **27**:53-8.
- Schell RF, Chan JK, Le Frock JL. Endemic syphilis: passive transfer of resistance with serum and cells in hamsters. *J Infect Dis* 1979; **140**:378-84.
- Soltani K, Aronson IK, Brickman F, Lorinez AL. Detection by direct immunofluorescence of antibodies to *Treponema pallidum* in cutaneous infiltrates of rabbit syphilomas. *J Infect Dis* 1978; **138**:222-5.
- Titus RG, Weiser RS. Experimental syphilis in the rabbit. Passive transfer of immunity with immunoglobulin G from immune serum. *J Infect Dis* 1979; **140**:904-13.
- Turner TG, Hollander DH. Biology of the treponematoses. *World Health Organisation Monograph Series No 35*. Geneva: WHO, 1957.
- Baseman JB, Hayes NS, Spitznagel JK, Hayes EC. Virulence determinants among the spirochetes. In: Schlessinger D, ed. *Microbiology* 1979. Washington DC: American Society for Microbiology, 1979;203-8.
- Baseman JB, Hayes EC. Molecular characterization of receptor-binding proteins and immunogens of virulent *Treponema pallidum*. *J Exp Med* 1980; **151**:573-86.
- Fitzgerald TJ, Johnson RC, Miller JN, Sykes JA. Characterization of the attachment of *Treponema pallidum*, (Nichols strain) to cultured mammalian cells and the potential relationship of attachment to pathogenicity. *Infect Immun* 1977; **18**:476-8.
- Fitzgerald TJ, Johnson RC, Sykes JA, Miller JN. Interaction of *Treponema pallidum* (Nichols strain) in tissue cultures: effect of oxygen, reducing agents, serum supplements, and different cell types. *Infect Immun* 1977; **15**:444-52.
- Fitzgerald TJ, Miller JN, Sykes JA. *Treponema pallidum* (Nichols strain) in tissue cultures: cellular attachment, entry, and survival. *Infect Immun* 1975; **11**:1133-40.
- Hayes NS, Muse KE, Collier AM, Baseman JB. Parasitism by virulent *Treponema pallidum* of host-cell surfaces. *Infect Immun* 1977; **17**:174-86.
- Alderete JF, Baseman JB. Characterization of the surface of virulent *Treponema pallidum*. *Infect Immun* 1980; **30**:814-23.
- Alderete JF, Baseman JB. Surface-associated host proteins on virulent *Treponema pallidum*. *Infect Immun* 1979; **26**:1048-56.
- Baseman JB, Hayes NS. Protein synthesis by *Treponema pallidum* extracted from infected rabbit tissue. *Infect Immun* 1974; **10**:1350-5.
- Graves S, Alden J. Limited protection of rabbits against infection with *Treponema pallidum* by immune rabbit sera. *Br J Vener Dis* 1979; **55**:399-403.
- Izzat NN, Knox JM, Werth JA, Dacres WG. Evolution of syphilitic chancres with virulent *Treponema pallidum* in the rabbit. *Br J Vener Dis* 1970; **47**:67-72.
- Kessler SW. Cell membrane antigen isolation with staphylococcal protein A-antibody adsorbent. *J Immunol* 1976; **117**:1482-90.
- Bonner WM, Laskey RA. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur J Biochem* 1974; **46**:83-8.
- Wilkinson BJ, Sisson SP, Kim Y, Peterson PK. Localization of the third component of complement on the cell wall of encapsulated *Staphylococcus aureus* M: implication for the mechanism of resistance to phagocytosis. *Infect Immun* 1979; **26**:1159-63.
- Pavia CS, Folds JD, Baseman JB. Cell-mediated immunity during syphilis. *Br J Vener Dis* 1977; **54**:144-50.
- Maret SM, Baseman JB, Folds JD. Cell-mediated immunity in *Treponema pallidum*-infected rabbits: in-vitro response of splenic and lymph node lymphocytes to mitogens and specific antigen. *Clin Exp Immunol* 1980; **39**:38-43.
- Ware JB, Folds JD, Baseman JB. Modulation of normal lymphocyte stimulation by serum from *Treponema pallidum*-infected rabbits. *Cell Immunol* 1980; **53**:29-36.
- Brause GD, Roberts RB. Attachment of virulent *Treponema pallidum* to human mononuclear phagocytes. *Br J Vener Dis* 1978; **54**:218-24.